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(54) Preparation of pure human tumor necrosis factor and hybridomas producing monoclonal antibodies to human tumor necrosis factor.

(57) Human TNF(LuKII) having a specific activity of at least 1.5×10^5 units per milligram of total protein is produced by contacting a TNF-containing protein composition, which has been harvested from human cell lines of hematopoietic origin or recombinant origin, in separate adsorption stages with glass beads, lentil lectin bound to Sepharose, and procion

red agarose, thereby selectively to adsorb TNF in each stage, while leaving some impurities unadsorbed, each contact stage being followed by contact of the adsorbent with an eluant thereby to obtain a solution of more highly purified TNF after each stage. The purified human TNF(LuKII) is used to produce monoclonal antibodies against TNF(LuKII)

and such antibodies can be used to assay samples
for the presence of TNF(LuKII).

PREPARATION OF PURE HUMAN TUMOR NECROSIS FACTOR AND HYBRIDOMAS PRODUCING MONOCLONAL ANTIBODIES TO HUMAN TUMOR NECROSIS FACTOR

BACKGROUND OF THE INVENTION

The present invention relates to a sequential chromatographic procedure for the purification of a human tumor necrosis factor, produced by the LuKII cell line, termed TNF(LuKII), the characterization of TNF(LuKII) and hybridoma cell lines producing monoclonal antibodies to TNF(LuKII).

It has been reported that during certain bacterial infections, for example, staphylococcal and streptococcal, there sometimes occurs a concomitant regression of human tumors.

Coley and others treated human malignancies with heat-killed bacterial vaccines and obtained positive results in some patients.

The presence of a tumor inhibitory factor in the sera of mice infected with bacillus Calmette-Guerin (BCG) and subsequently injected with endotoxin was reported by E.A. Carswell et al, Proc. Natl. Acad. Sci. USA, 72, 3666-3670 (1975). This sera has been observed to cause the hemorrhagic necrosis and regression of certain mouse tumors *in vivo*. This sera was also found to have cytotoxic/cytostatic effects on mouse and human tumor cells *in vitro* (E.A. Carswell et al, supra; L. Helson, et al, Nature (London), 258, 731-732 - (1975); D.N. Mannel et al, Infect. Immun., 28, 204-211 (1980); F.C. Kull and P. Cuatrecasas, J. Immunol., 126, 1279-1283 (1981); K. Haranaka and N. Satomi, Jpn. J. Exp. Med., 51, 191-194 (1981)). A similar factor was found to be induced in rats (Carswell et al, supra) and rabbits (Carswell et al, supra, N. Matthews and J.F. Watkins, Br. J. Cancer, 38, 302-309 (1978); J.M. Ostrove and G.E. Gifford, Proc. Soc. Exp. Biol. Med., 160, 354-358 - (1979)).

The antitumor factor present in the sera of animals sensitized to BCG or other immunopotentiating agents, such as Corynebacterium parvum, Malaria or Zymosan (yeast cell wall), and then challenged with endotoxin has been termed tumor necrosis factor (TNF).

Biochemical studies have indicated that mouse serum TNF is a glycoprotein and that its activity is associated with both high molecular weight components, e.g., M, 150,000, (Kull and Cuatrecasas, supra and S. Green et al, Proc. Natl. Acad. Sci. USA, 73, 381-385 (1976)) and components in the M, 40,000-60,000 range, (D.N. Mannel et al, supra; Kull and Cuatrecasas, supra; and Haranaka, supra). The molecule is stable when frozen, preferably below -70°C. Its activity is destroyed at 70°C for 30 minutes. It is pyrogenic in rabbits in a range

from 5-500 microgram/kg and non-pyrogenic at 5 microgram/kg. TNF in rabbit serum has also been reported to have a molecular weight of 39,000, (N. Matthews et al, Br. J. Cancer, 42, 416-422 (1980)), and 67,000, (M.R. Ruff and G.E. Gifford, J. Immunol., 125, 1671-1677 (1980)).

Studies have indicated that both *in vivo* and *in vitro* activities of mouse TNF appear to be a property of the same molecule. The cellular source of TNF in the mouse was initially assumed to be the macrophage, because the agents used to prime for TNF production cause massive hyperplasia of macrophages in liver and spleen (Carswell et al, supra). From studies of macrophage-rich cell populations *in vitro*, (N. Matthews, Br. J. Cancer, 38, 310-315 (1978) and D.N. Mannel et al, Infect. Immun., 30, 523-530 (1980)) a similar conclusion was reached with regard to the source of mouse and rabbit TNF. Direct evidence that macrophages are at least one cell type in the mouse capable of producing TNF comes from studies with cloned lines of mouse histiocytomas (D.N. Mannel et al, supra and unpublished data). These cells constitutively produce low levels of TNF that are greatly increased after exposure to endotoxin.

B.D. Williamson et al, Proc. Natl. Acad. Sci. USA, 80, 5397-5401 (1983), described the capacity of human cell lines of hematopoietic origin, e.g., B-cell lines, to produce a factor with TNF activity. The product of one of the B-cell lines (LuKII) was chosen for detailed studies. Evidence demonstrating that this molecule is a human TNF included the following: (1) the anticellular response of a panel of human cell lines to human TNF, e.g., TNF(LuKII), or mouse TNF are indistinguishable and can be potentiated in a synergistic fashion by interferon, - (2) mouse L cells made resistant to mouse TNF are resistant to human TNF, e.g., TNF (LuKII), (3) mouse L cells made resistant to human TNF, e.g., TNF(LuKII) are resistant to mouse TNF, and (4) human TNF, e.g., TNF(LuKII), causes hemorrhagic necrosis of Meth A sarcoma in the standard *in vivo* TNF assay, B.D. Williamson, supra.

Heretofore, there have been no known purification methodologies to obtain pure TNF(LuKII) which are rapid and efficient, for example, which do not require dialysis which may involve serious losses of material. At the present time there is also no hybridoma in existence which produces a monoclonal antibody to the pure TNF(LuKII), which would be extremely useful for purification, diagnostic, and perhaps therapeutic purposes.

SUMMARY OF THE INVENTION

It is accordingly an object of the present invention to provide a process for the purification of TNF(LuKII) which yields both good recoveries of TNF activity and high specific activity material. The pure TNF(LuKII) can be used therapeutically.

It is another object of the present invention to isolate this TNF(LuKII) in a form suitable for producing hybridoma cell lines which produce monoclonal antibodies to TNF(LuKII), to allow for the rapid purification and detection of the presence of TNF(LuKII) for diagnostic purposes.

This and other objects and advantages are realized in accordance with the present invention pursuant to which a purified TNF(LuKII), i.e., a human TNF having an activity of at least 1.5×10^5 units per milligram of total protein is obtained by contacting a TNF-containing protein composition, which has been harvested from human cell lines - (e.g., human cell lines of hematopoietic origin, particularly B-cell lines such as the LuKII cell line) or produced by recombinant technologies, in separate adsorption stages with glass beads, lentil lectin bound to Sepharose, and procion red agarose, thereby selectively to adsorb TNF in each stage while leaving some impurities unadsorbed, each contact stage being followed by contact of the adsorbent with an eluant thereby to obtain a solution of more highly purified TNF after each stage. In a preferred process for obtaining purified TNF, the glass beads are the first stage adsorbent, the Sepharose-bound lentil lectin is the second stage adsorbent and the procion red agarose is the third stage adsorbent.

The purification process of the present invention allows the TNF(LuKII) eluted from one column to be applied, either directly or after dilution, onto the next column, thereby eliminating any need for dialysis and thus avoiding the losses associated with dialysis.

The TNF which is purified to obtain the TNF of the invention can be harvested from human cell lines of hematopoietic origin, particularly B-cell lines. Recombinant methodologies can also be used to produce a TNF-containing composition. For example, the gene responsible for TNF(LuKII) production can be implanted in *E. coli* which, in turn, produce TNF(LuKII) in large quantities. The recombinant techniques used are known (e.g., T. Maniatis et al, Molecular Cloning, Cold Spring Harbor Laboratory, 1982).

The purified TNF(LuKII) of the invention comprises a plurality of fractions of protein molecules of different molecular weights, each of which, it is believed, has TNF activity. More particularly, the TNF(LuKII) comprises at least three fractions. Even more particularly, the TNF(LuKII) comprises seven

fractions, each of which, it is believed, has TNF activity.

The purified TNF(LuKII) of the invention may have different numbers of protein fractions, each fraction having a different molecular weight. For example, the TNF(LuKII) of the invention may have two fractions, for example, corresponding to the molecular weights of, for example, 70,000 and 25,000 daltons, respectively; or, the TNF(LuKII) of the invention may have three fractions, for example, corresponding to the molecular weights of, for example, 70,000, 25,000 and 19,000 daltons, respectively; or the TNF(LuKII) of the invention may have seven fractions corresponding to the molecular weights of 80,000; 70,000; 43,000; 25,000; 23,000; 21,000; and 19,000 daltons, respectively.

The TNF(LuKII) of the invention is further characterized in having a specific activity of at least 1.5×10^5 units per milligram of total protein, e.g., 1.5×10^5 to 1.5×10^6 units per milligram of total protein, more particularly, 1.5×10^6 to 1.5×10^7 , and even more particularly, about 1.5×10^7 units per milligram of total protein.

The TNF(LuKII) of the invention can contain chemically bound carbohydrate moieties, for example; glycosyl groups:

As described above, the inventive TNF(LuKII) comprises a plurality of protein fractions, e.g., at least three of and as many as seven or perhaps even more fractions which it is believed will have TNF activity. No matter how many fractions are obtained, each such protein fraction constituting the TNF(LuKII), is disruptable upon contact with enzymes such as trypsin or chymotrypsin to produce tryptic or chymotryptic digested proteins. At least one such protein fragment generated by enzyme digestion of each given protein fraction and at least one fragment generated by digestion of any other protein fraction or fractions, migrate to the same position following two dimensional analysis on a cellulose coated glass plate. Fragments generated following enzyme digestion of 70K and 25K proteins, for example, migrate to the same position on two dimensional peptide mapping analysis and are thus related. In particular, two of the fragments generated by trypsin digestion of the 70K protein and two of the fragments generated by digestion of the 25K protein migrate to the same position upon two dimensional peptide mapping analysis, i.e., peptide homology. Peptide mapping analysis also shows that the 43K, 25K, 23K, 21K and 19K proteins, for example, are related, as are the 80K and 70K proteins.

In accordance with another aspect of the invention, several protein fractions of the inventive TNF(LuKII), having a different molecular weight, are recognized by monoclonal antibodies of the inven-

tion, produced by hybridomas, for example, ATCC HB 8887, deposited on August 14, 1985.

In accordance with another aspect of the invention, the TNF(LuKII), which has a specific activity of 1.5×10^5 to 1.5×10^6 units per milligram of total protein, is *in vitro* cytotoxic or cytostatic, or has no effect on various human tumor cell lines.

For example, cytotoxic effects of hTNF on the following cell lines have been shown: SK-MG-4 - (astrocytoma), MCF-7 (breast cancer), BT-20 - (breast cancer), SK-BR-3 (breast cancer), ME-180 - (cervix cancer), SK-CO-1 (colon cancer) and RPMI 7931 (melanoma).

Cytostatic effects of hTNF on the following cell lines, for example, have also been shown: SK-LU-1 (lung cancer), RPMI 4445 (melanoma), SK-MEL-29 (melanoma), SK-MEL-109 (melanoma) and SK-OV-3 (ovary cancer).

The present invention also concerns a method of producing monoclonal antibodies against TNF(LuKII) by propagating a hybridoma cell line which secretes such monoclonal antibody and harvesting secreted antibody.

Another aspect of the invention relates to a method of assaying a sample for the presence of TNF(LuKII), which comprises contacting the sample with a monoclonal antibody against TNF(LuKII), separating the antibody with any TNF(LuKII) which has combined therewith, and assaying the separated antibody for the presence of any TNF(LuKII) combined therewith. For example, using monoclonal antibodies of the invention, both enzyme linked immunosorbent assays and radioimmunoassays, can be used to detect the presence of TNF(LuKII) in a sample.

Two particular methods of assaying a sample for the presence of TNF(LuKII) are as follows:

(1) Competitive Method:

An antibody, e.g., a monoclonal antibody, having an affinity to TNF(LuKII) is coated on a substrate, e.g., glass beads. Such coated beads are contacted with an unknown sample suspected of containing TNF(LuKII). Labelled TNF, e.g., radiolabelled or enzyme labelled, is allowed to contact the coated bead and unknown sample. Washing is then conducted. The amount of labelled TNF(LuKII) that binds is effected by any TNF(LuKII) in the unknown sample (unlabelled TNF(LuKII)). If there is no TNF(LuKII) in the sample, all the labelled TNF(LuKII) binds. The more TNF(LuKII) in the sample, the less labelled TNF(LuKII) binds. To determine the binding of the labelled TNF(LuKII), counts are taken if the label is a radiolabel and if an enzyme label is utilized, the

enzymatic activity is determined.

(2) Non-Competitive Method:

A substrate, e.g. polystyrene beads, is coated with an antibody, e.g., a monoclonal antibody having an affinity for TNF(LuKII). The coated beads are then contacted with an unknown sample suspected of containing TNF(LuKII). The bead is then washed and a labelled antibody having an affinity for TNF(LuKII) is then allowed to contact the bead. Washing is then conducted. The amount of antibody bound to the bead is a reflection of the TNF(LuKII) in the sample. If there is no TNF(LuKII) in the sample, there is no binding of the antibody. The more TNF(LuKII) in the sample, the greater the binding of the antibody. The TNF(LuKII) acts as a bridge between the labelled antibody and the unlabelled antibody.

The monoclonal antibodies of the invention can also be used to obtain the purified TNF(LuKII) of the invention. For example, a monoclonal antibody can be immobilized on a solid support, e.g., attached to Sepharose beads in a column, and the crude TNF(LuKII) passed through the column and the column washed with buffers. Immobilized TNF(LuKII) bound to the monoclonal antibody is then dissociated therefrom by using a dissociation buffer.

The invention also relates to the treatment of a patient having a tumor by administering to such patient a tumor-necrotic or a regression-effective amount of TNF(LuKII) of the invention, i.e., having a specific activity of at least 1.5×10^5 units per milligram of protein, more particularly, 1.5×10^5 to 1.5×10^6 units per milligram protein, more particularly, 1.5×10^6 to 1.5×10^8 and even more particularly, about 1.5×10^7 units per milligram of total protein.

DESCRIPTION OF THE DRAWINGS

The invention will be further described with reference to the accompanying drawings wherein:

Figure 1a is a chromatograph of media conditioned by the LuKII cell line, containing TNF(LuKII), on a controlled pore glass column. LuKII culture media (8 liters) containing 200 units/mL of TNF(LuKII) was applied to a controlled pore glass column (50mL) equilibrated with phosphate buffered saline (20 mM sodium phosphate, pH 7.0, 0.15 M NaCl) (PBS). The column was washed with the following buffers in sequence: PBS (75 mL), PBS containing 20% ethylene glycol (v/v) (E) (225 mL), PBS (120 mL), 20 mM sodium phosphate, pH 7.0, containing 1.15 M NaCl (PBS + 1 M

NaCl) (E₁) (175 mL), PBS (50 mL), 5 mM sodium phosphate, pH 6.8, (E₂) (225 mL), and 5 mM sodium phosphate, pH 6.8, containing 5% triethylamine (v/v) (E₃) (150 mL). Eluted fractions were collected in polypropylene bottles. The material eluted with the E₄ buffer was collected in 50 mL aliquots.

Figure 1b is a lentil lectin Sepharose column chromatograph of TNF(LuKII) purified by controlled pore glass chromatography. 150 mL of the partially purified TNF(LuKII) eluted from the controlled pore glass column was loaded onto a lentil lectin sepharose column (10 mL) equilibrated with PBS. The column was washed sequentially with PBS (40 mL), PBS + 1 M NaCl (E₁) (24 mL), and PBS + 1 M NaCl containing 0.2 M α -methyl-D-mannoside - (E₂) (60 mL). The material eluted with the α -methyl-D-mannoside containing buffer was collected in 10 mL aliquots.

Figure 1c is a procion red agarose column chromatograph of the TNF(LuKII) purified sequentially first on a controlled pore glass column and then on a lentil lectin sepharose column. 60 mL of partially purified TNF(LuKII) eluted from the lentil lectin column was diluted 1:1 with PBS and loaded onto a procion red agarose column (4 mL) equilibrated with 20 mM sodium phosphate, pH 6.8, 0.65 M NaCl (PBS + 0.5 M NaCl). The column was washed with the following buffers in sequence: PBS + 0.5 M NaCl (E₁) (30 mL), PBS + 1 M NaCl (E₂) (8 mL), PBS (8mL), PBS containing 50% ethylene glycol (v/v) (E₃) (8 mL), PBS (8mL), 0.1 M Tris-HCl, pH 9.4 + 0.1 M NaCl (E₄) (8 mL), and 0.1 M Tris-HCl, pH 9.4 + 0.1 M arginine (E₅) (24 mL). The material eluted with the 0.1 M Tris-HCl, pH 9.4 + 0.1 M arginine buffer was collected in 4 mL aliquots.

Figure 2 is a graph indicating the isoelectric point of TNF(LuKII). A 60 μ L sample of purified TNF(LuKII) containing 1500 units was applied to a pH 3.5 -9.5 ampholine gel.

Figure 3 is an autoradiograph resulting from NaDodSO₄ polyacrylamide gel electrophoresis (PAGE) of purified ¹²⁵I-labeled TNF(LuKII). TNF(LuKII) was iodinated and fractionated by NaDodSO₄/PAGE. Autoradiographs were developed for 18 hours (lane 1) and 0.5 hour (lane 2). The following proteins provided Mr markers: myosin - (200,000), β -galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin - (67,000), ovalbumin (43,000), α -chymotrypsinogen (25,700), β -lactoglobulin (18,400), lysozyme - (14,300) and cytochrome C (12,300).

Figure 4 is a graph showing recovery of TNF(LuKII) activity after NaDodSO₄/PAGE fractionation of TNF(LuKII). A sample of TNF(LuKII) containing 6000 units adjusted to contain 0.1% NaDodSO₄ and 0.1 M β -mercaptoethanol was applied to a

12% polyacrylamide gel. Following electrophoresis, the gel was sliced and activity eluted and assayed. In an adjacent track, ¹²⁵I-labeled TNF(LuKII) was fractionated and autoradiographed to determine the Mr of the TNF(LuKII) active fractions.

Figures 5a and 5b are tryptic peptide maps of ¹²⁵I-labeled proteins in purified TNF(LuKII). ¹²⁵I-labeled TNF(LuKII) was fractionated on NaDodSO₄/PAGE and individual protein bands present in gel slices were incubated overnight in the presence of 50 μ g/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone-(TPCK)-treated trypsin. The individual gel slices were then washed with water and 10,000 CPM samples of each lyophilized to dryness. These samples were dissolved in a buffer containing formic acid and acetic acid and applied to cellulose pre-coated glass TLC plates at the origin (x). Electrophoresis was performed from right to left followed by ascending chromatography in a buffer containing butanol, pyridine and acetic acid. Autoradiographs of the tryptic maps of the Mr 80,000, 70,000, 43,000, 25,000, 23,000, 21,000 and 19,000 proteins are presented in Fig. 5a. Fig. 5b shows tryptic maps of the Mr 25,000, 70,000 and a mixture of the 25,000 and 70,000 proteins.

Figures 6a and 6b are chymotryptic peptide maps of ¹²⁵I-labeled proteins in purified TNF(LuKII). ¹²⁵I-labeled proteins present in the purified TNF(LuKII) preparation were processed as described in Figure 5, except that for this digestion, 50 μ g/mL of N- α -tosyllysine chloromethyl keton(TLCK)-treated chymotrypsin was used. Autoradiographs of the chymotryptic maps of the Mr 80,000, 70,000, 43,000, 25,000, 23,000, 21,000 and 19,000 proteins are presented in Fig. 6a. Fig. 6b shows chymotryptic maps of the Mr. 25,000, 70,000, and a mixture of the Mr 25,000 and 70,000 proteins.

Figure 7 shows the results of immunoblotting analysis of TNF(LuKII) with T1-18 mouse monoclonal antibody. A sample of TNF(LuKII) containing 10,000 units of TNF(LuKII) was fractionated by NaDodSO₄/PAGE. Fractionated proteins were transferred to a nitrocellulose membrane and processed.

The invention will be further described in the following illustrative non-limiting examples wherein all parts are by weight unless otherwise expressed.

DETAILED DESCRIPTION OF INVENTION

Examples

Example 1:

Production of Tumor Necrosis Factor

LuKII cells (a cell line of B-cell origin) were obtained as described in Pickering, L. A., Kronenberg, L. H. & Stewart, W.E., II, Proc. Natl. Acad. Sci USA, 77, 5938-5942 (1980), and were cultured in the following manner in order to obtain human tumor necrosis factor produced by the LuKII cells - [i.e., TNF(LuKII)]:

LuKII cells (8×10^5 cells/ml) were placed in RPMI 1640 media containing 8% fetal calf serum - (FCS) with 10 ng/ml of mezerein (L.C. Services, Woburn, MA) for 48 hours. The cells were then removed from the media by centrifugation, resuspended in fresh RPMI 1640 media lacking any protein supplement and allowed to incubate for an additional 48 hours. Cells were removed by centrifugation, and the culture media used as the source of TNF(LuKII).

Example 2:

Purification of TNF(LuKII)

Human TNF(LuKII) was purified sequentially using controlled pore glass, lentil lectin Sepharose and procion red agarose column chromatography as follows (all affinity chromatography procedures were carried out at room temperature and all column fractions were collected in polypropylene tubes or bottles):

The TNF(LuKII) culture fluid was applied to a column of controlled pore glass-350 (Electronucleonics, Fairfield, N.J.) which bound all

of the TNF activity. The column was washed with several buffers in sequence (as described in the legend to Figure 1a) and then the TNF activity was eluted with a 5mM sodium phosphate buffer, pH 6.8, containing 5% triethylamine (Figure 1a). The eluted TNF was then applied to a lentil lectin Sepharose column (Pharmacia, Piscataway, N.J.), which was then washed first with phosphate buffered saline (PBS) followed by a 0.02 M sodium phosphate buffer, pH 6.8, containing 1.15 mM NaCl - (PBS + 1 M NaCl). TNF activity was then eluted from this column with PBS + 1 M NaCl buffer containing 0.2 M α -methyl-D-mannoside (Figure 1b). All TNF activity bound to the lentil lectin Sepharose column and 39% of the activity was recovered in the α -methyl-D-mannoside-containing buffer. The further washing of the column with the above buffer containing 50% ethylene glycol eluted only a small amount of TNF activity. TNF from the lentil lectin column was then diluted 1:1 with PBS and loaded onto a procion red agarose column - (Bethesda Research Laboratory, Bethesda, Maryland). The column was washed sequentially with several buffers (as described in the legend to Figure 1c) which removed protein having no TNF activity. The column was then washed with 0.1 M tris-HCl, pH 9.4, containing 0.1 M arginine. The TNF activity was eluted with this buffer, yielding TNF with a specific activity of 1.5×10^7 units/mg of protein. Table 1 summarizes the purification scheme for TNF(LuKII) with specific activities of the resulting fractions.

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Table I. Purification of TNF (LuKII)

Column	Load (units)	Load (Specific Activity) μmg	Recovery (units)	Recovery (Specific Activity) μmg	% Recovery	Fold Purification
Controlled Fore Glass	1.6×10^6	5.3×10^3	9.6×10^5	3.8×10^5	60%	72X
Lentil Lectin Sepharose	9.6×10^5	3.8×10^5	6.3×10^5	1.3×10^6	39%	245X
Pirccion Red Agarose	6.3×10^5	1×10^6	6.3×10^5	1.5×10^7	39%	2830X

Example 3 :Preparation of Monoclonal Antibody to TNF(LuKII)

BALB/c mice were injected with 1600 units of purified TNF(LuKII), with a specific activity of $1.5 \times$

10^7 units/mg. For the initial injection, TNF(LuKII) was mixed with Freund's complete adjuvant (1:1) and injected subcutaneously. Subsequent injections were given intraperitoneally in the absence of adjuvant. Serum antibody to TNF(LuKII) was determined by an enzyme linked immunosorbent assay (ELISA) in which the TNF(LuKII) was bound to polystyrene plates. After nine immunizations over a

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period of seven months, the spleen of one mouse with a high titer antibody directed against TNF-(LuKII) was removed and fused with cells of the P₃U₁ mouse plasmacytoma cell line. Resulting clones were screened for their ability to bind TNF-(LuKII) in ELISA assays. A hybridoma (designated T1-18) producing antibody reactive with TNF-(LuKII) was isolated and subcloned. This hybridoma grown in tissue culture media as well as in ascites served as a source of TNF(LuKII) antibody. The hybridoma has been deposited with the ATCC as HB 8887, deposited on August 14, 1985.

Example 4:

Biochemical Characterization of Purified TNF-(LuKII)

To initiate the characterization of the purified TNF(LuKII), isoelectrofocusing was performed using Ampholine Pagplates (pH 3.5 to 9.5) LKB Instruments, Gaithersburg, MD). The gels were run at 30 watts for 1.5 hours at which time the pH gradient was measured. Human TNF was found to have an isoelectric point of approximately 6.7 (see Figure 2). The gel was sliced into 18 equal pieces. The gel fractions were incubated for 18 hours in Eagle's Minimum Essential Medium (MEM) containing 10% fetal calf serum, and fractions were assayed for the presence of TNF(LuKII) *in vitro*.

Purified TNF(LuKII) was tested and was observed to cause hemorrhagic necrosis of the Meth A mouse sarcoma in the standard *in vivo* TNF assay. The *in vivo* TNF assay was performed as described (Williamson B. D. *et al*, *supra*). Essentially, (BALB/c × C57BL/6)F₁ female mice were injected intradermally with 5×10^5 Meth A BALB/c sarcoma cells. After 7 days (tumor size approximately 7 mm average diameter), mice received either a single intravenous or intratumoral injection of the TNF(LuKII) preparation. After 24 hours, tumor hemorrhagic necrosis was scored according to Carswell *et al*, *supra*. TNF(LuKII) causes hemorrhagic necrosis of Meth A sarcoma after intratumoral or intravenous injection and total tumor regression has been observed in some treated mice.

The *in vitro* assay for TNF was performed in 96-well microtiter plates. Serially diluted fractions were sterilized by ultraviolet radiation and the TNF-sensitive L cells (for example, derived from mouse L cells obtained from American Type Culture Collection) was added to each well at a density of 2×10^4 cells/well in 100 μ L. After two days at 37°C, the plates were examined by phase-contrast microscopy and the percentage of dead cells was determined. The unitage of the sample was calculated as the reciprocal of the highest dilution that killed

50% of the cells. All TNF assays were run in parallel with a laboratory standard and titers are expressed in laboratory units.

Purified TNF(LuKII) was iodinated as follows:

TNF(LuKII) was labeled with ¹²⁵I using 1,3,4,6-tetrachloro-3 α , 6 α -diphenylglycouril (Iodo-gen, Pierce Chemical Co., Rockford, IL) as follows.

Polypropylene tubes were coated with 100 μ g of Iodo-gen (dissolved in chloroform) by evaporation of the solvent. A 2 ml sample of TNF(LuKII) (50,000 units/ml) with a specific activity of 1.5×10^7 units/mg of protein was incubated for 25 minutes at room temperature in an Iodo-gen coated tube containing 2 mCi of ¹²⁵I. The labeled protein was then separated from the unbound ¹²⁵I using a P-4 column (Bio-Rad, Richmond, CA) equilibrated with phosphate buffered saline (PBS) containing 50 μ g/mL of cytochrome-C. The iodinated material eluted in the void volume of the column was divided into aliquots and stored at -80°C.

Following iodination, the radioactively labelled proteins present in the preparations were analyzed by NaDodSO₄/polyacrylamide gel electrophoresis (PAGE) which was performed in 18 cm slab gels according to conditions described by Laemmli, U.K. (1970), *Nature* (London), **227**, 680-685.

All protein determinations were made using the Bio-Rad dye reagent (Bio-Rad, Richmond, CA), using bovine serum albumin as a standard.

As can be seen in Fig. 3, the purified preparation of the TNF(LuKII) contained seven protein bands with molecular weights of 80,000, 70,000, 43,000, 25,000, 23,000, 21,000 and 19,000 daltons (80K, 70K, 43K, 25K, 23K, 21K, 19K). The same seven protein bands were observed when non-labeled purified TNF(LuKII) was fractionated by NaDodSO₄/PAGE and examined by the silver staining of the gel. The Mr 80,000 and 70,000 forms were eluted from the gels and re-analyzed by NaDodSO₄/PAGE. They migrated once again to the Mr 70,000 -80,000 region and no smaller molecular weight components were observed. In further experiments, purified TNF(LuKII) was boiled in NaDodSO₄, urea and β -mercaptoethanol and the same characteristic seven bands were found.

In order to ascertain which of the protein bands present in the purified TNF(LuKII) preparations was responsible for TNF activity, parallel samples of purified TNF(LuKII), one ¹²⁵I-labeled and one unlabeled, were treated with NaDodSO₄ (0.1%) and β -mercaptoethanol (0.1 M) and then fractionated by NaDodSO₄/PAGE. Upon completion of the electrophoresis, the lane containing the unlabeled TNF-(LuKII) was cut into 4.4 mm slices and the slices put into Eagle's minimal essential media (MEM) containing 10% fetal calf serum (FCS). The proteins were then eluted from each gel slice by

overnight incubation at 4°C in MEM containing FCS. The samples were assayed for the presence of TNF as described above.

The parallel lane containing ¹²⁵I-labeled TNF-(LuKII) was dried immediately following the completion of the electrophoresis and the protein bands located by autoradiography. As can be seen in Fig. 4, TNF activity was recovered from the gel at molecular weights of approximately 70,000 and 19,000 to 25,000 daltons, corresponding to ¹²⁵I-labeled protein bands at these positions. In experiments in which β-mercaptoethanol was not added to the TNF(LuKII) sample before NaDodSO₄/PAGE, TNF activity was also recovered at the 70,000 and 19,000 to 25,000 molecular weight range.

Since it was observed that following NaDodSO₄/PAGE, TNF activity was recovered at the 70,000 as well as the 19,000 to 25,000 dalton region, the relatedness of the various proteins present in the purified TNF(LuKII) preparations was then examined. To accomplish this, two dimensional chymotryptic and tryptic peptide mapping analyses of the individual protein bands present in the most purified TNF(LuKII) preparations were performed.

To perform the peptide mapping, an ¹²⁵I-labeled preparation of purified TNF(LuKII) was fractionated by NaDodSO₄/PAGE and individual bands (i.e., 80K, 70K, 43K, 25K, 23K, 21K, 19K) localized by autoradiography were cut from the gel and treated with either TPCK treated trypsin or TLCK treated chymotrypsin. Digested fractions were then analyzed according to the methods of J.H. Elder *et al.* Nature (London), 267, 23-28 (1977).

As seen in Fig. 5a, the tryptic peptide maps demonstrate that the 43K, 25K, 23K, 21K and 19K proteins are related and that the 80K and 70K proteins are related. To examine the relationship between the larger molecular weight proteins (e.g., 70K and 80K) and the smaller molecular weight proteins (e.g., 19 to 25K), tryptic digests of the 70K and 25K proteins were mixed so that they contained equal amounts of radioactivity and the mixture was analyzed by peptide mapping analysis. As seen in Fig. 5b, two of the fragments (termed A and B) generated by digestion of the 70K protein and two of the fragments generated by digestion of the 25K protein overlap, i.e., migrate to the same position upon peptide mapping analysis. A similar analysis was carried out using chymotrypsin as the proteolytic enzyme. As seen in Fig. 6a, the 43K, 25K, 23K, 21K and 19K proteins are related and the 80K and 70K proteins are related. To once again examine the relatedness between the smaller and larger molecular weight proteins, the chymotrypsin digests 25K and 70K proteins were mixed and the mixture analysed by peptide mapping analysis. As seen in Fig. 6b, at least three of

the fragments generated (termed A, B and C) following chymotrypsin digestion of the 25K protein migrate to the same position as three of the fragments generated by digestion of the 70K protein.

Further evidence for the relationships among the various proteins in purified TNF(LuKII) comes from immunoblotting analyses with T1-18 monoclonal antibody to TNF(LuKII). Western blotting analysis was performed essentially as described in Burnette, W.N. (1981), Anal.Biochem., 112, 195-203. Briefly, preparations of purified TNF(LuKII) were fractionated by NaDodSO₄/PAGE and the proteins present in the gel were transferred to nitrocellulose paper overnight at 100 mA. Following incubation of the nitrocellulose paper in a buffer containing bovine serum albumin (BSA), the nitrocellulose paper was exposed for two hours to 40 mL of T1-18 monoclonal antibody-containing culture medium. The nitrocellulose paper was then washed extensively and incubated overnight in 10 mM Tris-HCl, pH 7.4, + 0.9% NaCl containing 5% BSA and ¹²⁵I-labeled rabbit anti-mouse IgG. The nitrocellulose paper was further washed and exposed to X-ray film.

As seen in Fig. 7, exposure of this nitrocellulose paper to the X-ray film reveals that the monoclonal antibody to TNF(LuKII) reacts with proteins with molecular weights of 43K and 19K to 25K, thus showing shared determinants on the Mr 43,000 and 19-25,000 proteins. Antibody did not react with the higher molecular weight forms, even though these have been shown to be related to the Mr 43,000 and lower molecular weight components. This could be due to the inaccessibility of the determinant on the Mr 70,000 and 80,000 species. Thus, analysis indicates that there are a number of structural related proteins in purified TNF(LuKII) and that TNF activity is associated with nondissociable high molecular weight and low molecular weight forms. It is concluded that the seven proteins in the purified TNF(LuKII) are the products of related genes or products of a single gene that undergoes extensive processing.

Thus, using peptide map analyses, and a monoclonal antibody to human TNF in western blotting analysis it has been demonstrated that the various molecular weight proteins present in the purified TNF(LuKII) preparations are related.

Example 5:

The Relationship of Mouse TNF to TNF(LuKII).

A standard lot of partially purified mouse serum TNF with a specific activity of 2×10^4 units of TNF per mg of protein was used in these studies. A unit of TNF is defined as the amount of protein causing

killing of 50% of the L cells in the standard in vitro TNF assay.

To investigate further the relationship of mouse TNF to TNF(LuKII), L cells were made resistant to TNF(LuKII) by repeated passage in TNF(LuKII)-containing medium. TNF(LuKII)-resistant cells showed complete cross-resistance to mouse TNF. L cell lines made resistant to mouse TNF or to partially purified TNF(LuKII) are resistant to purified TNF(LuKII). TNF(LuKII) is cytotoxic to mouse L cells sensitive to mouse TNF.

It will be appreciated that the instant specification and claims are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departure from the spirit and scope of the present invention.

Claims

1. Human TNF(LuKII) having a specific activity of at least 1.5×10^5 units per milligram of total protein.

2. Human TNF(LuKII) according to claim 1, wherein said specific activity is 1.5×10^5 to 1.5×10^6 units per milligram of total protein.

3. Human TNF(LuKII) according to claim 1, wherein said specific activity is 1.5×10^6 to 1.5×10^7 units per milligram of total protein.

4. Human TNF(LuKII) according to claim 1, wherein said specific activity is about 1.5×10^7 units per milligram of total protein.

5. Human TNF(LuKII) according to claim 1, containing chemically bound carbohydrate moieties.

6. Human TNF(LuKII) according to claim 5, wherein said moieties comprise glycosyl groups.

7. Human TNF(LuKII) according to claim 1, comprising a plurality of fractions of protein molecules of different molecular weights.

8. Human TNF(LuKII) according to claim 1, wherein at least two fractions have peptide homology.

9. Human TNF(LuKII) according to claim 7, wherein said human TNF comprises seven fractions.

10. Human TNF(LuKII) according to claim 7, wherein said TNF(LuKII) has fractions corresponding to the following molecular weights:

70,000, 25,000 and 19,000 daltons.

11. Human TNF(LuKII) according to claim 7, wherein said TNF has fractions corresponding to the following molecular weights:

80,000 daltons

70,000 daltons

43,000 daltons

5 25,000 daltons

23,000 daltons

10 21,000 daltons and

19,000 daltons.

12. Human TNF(LuKII) according to claim 7, wherein several different protein fractions are recognized by a monoclonal antibody directed against human TNF(LuKII).

13. A process for producing human TNF(LuKII) according to claim 1, which comprises contacting a TNF(LuKII)-containing protein composition, which has been harvested from human cell lines of hematopoietic origin or from recombinant origin, in separate adsorption stages with glass beads, lentil lectin bound to Sepharose, and procion red agarose, thereby selectively to adsorb TNF(LuKII) in each stage while leaving some impurities unadsorbed, each contact stage being followed by contact of the adsorbent with an eluant thereby to obtain a solution of more highly purified TNF(LuKII) after each stage.

14. A process according to claim 13, wherein the glass beads are the first stage adsorbent, the Sepharose bound lentil lectin is the second stage adsorbent and the procion red agarose is the third stage adsorbent.

15. Hybridoma cell line ATCC HB 8887.

16. A monoclonal antibody or fragment thereof secreted by ATCC HB 8887.

17. A method of producing a monoclonal antibody against human TNF(LuKII), comprising

(a) propagating a hybridoma cell line which secretes such monoclonal antibody, and
(b) harvesting secreted antibody.

18. A monoclonal antibody produced by the process of claim 17.

19. A method of assaying a sample for the presence of TNF(LuKII), which comprises contacting the sample with a monoclonal antibody against human TNF(LuKII), separating the antibody with any TNF(LuKII) which has combined therewith, and assaying the separated antibody for the presence of TNF(LuKII) from the sample combined therewith.

20. In the treatment of a patient having a tumor by administering to the patient a tumor-necrotic effective amount of TNF or a tumor-regressive effective amount of TNF, the improvement which comprises employing as the TNF, TNF(LuKII) according to claim 1.

Fig. 1a

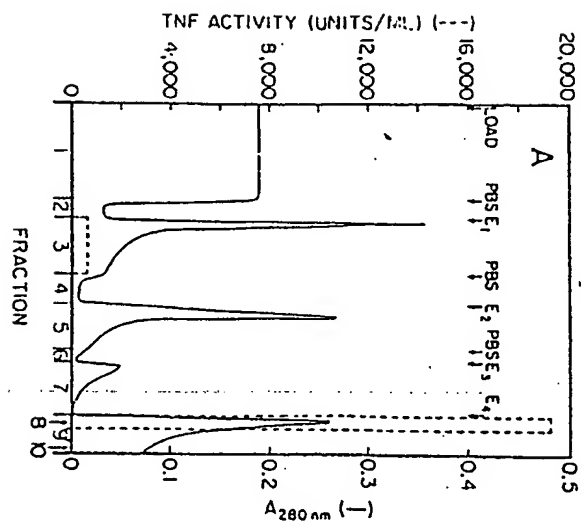


Fig. 1b

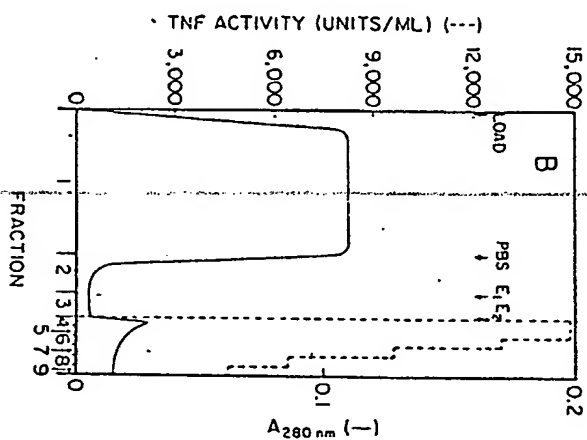


Fig. 1c

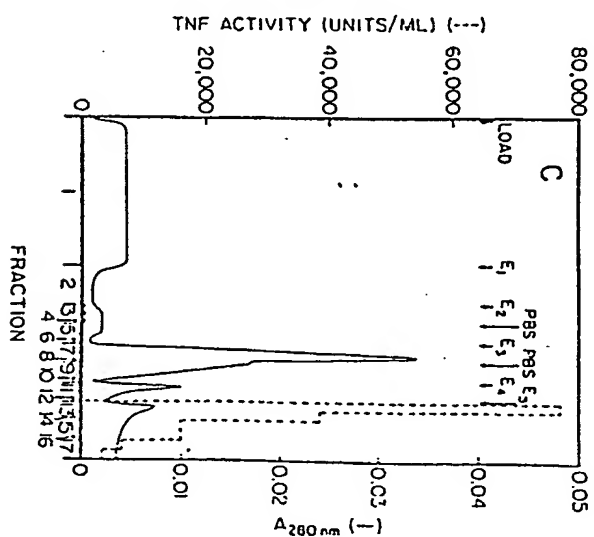


Fig. 2

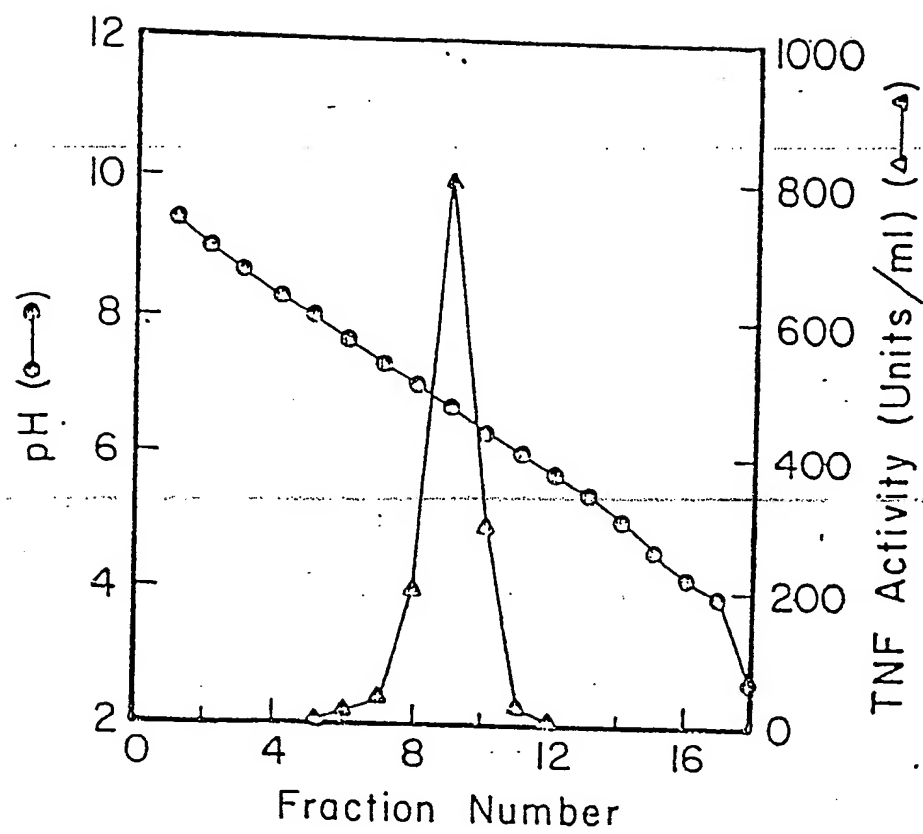


Fig. 3

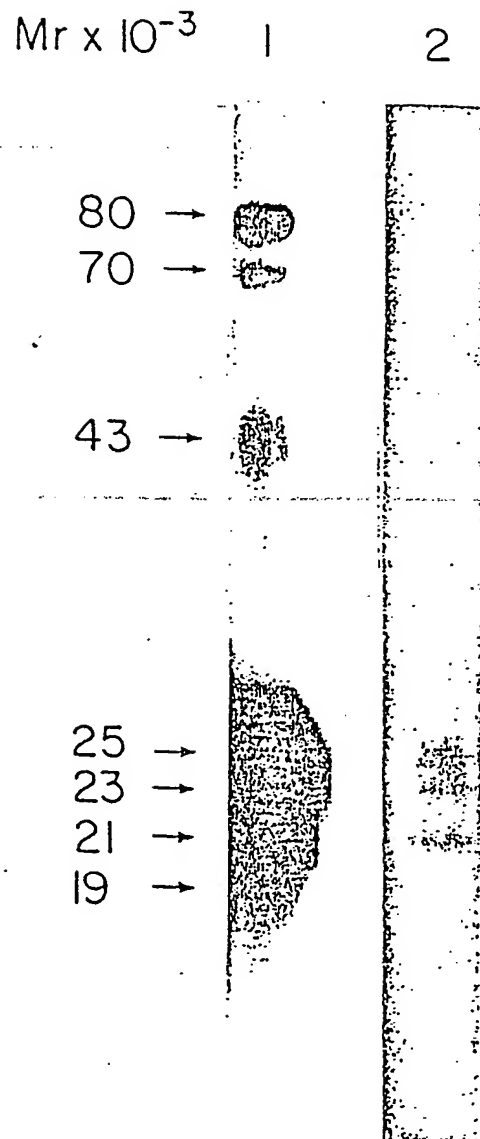
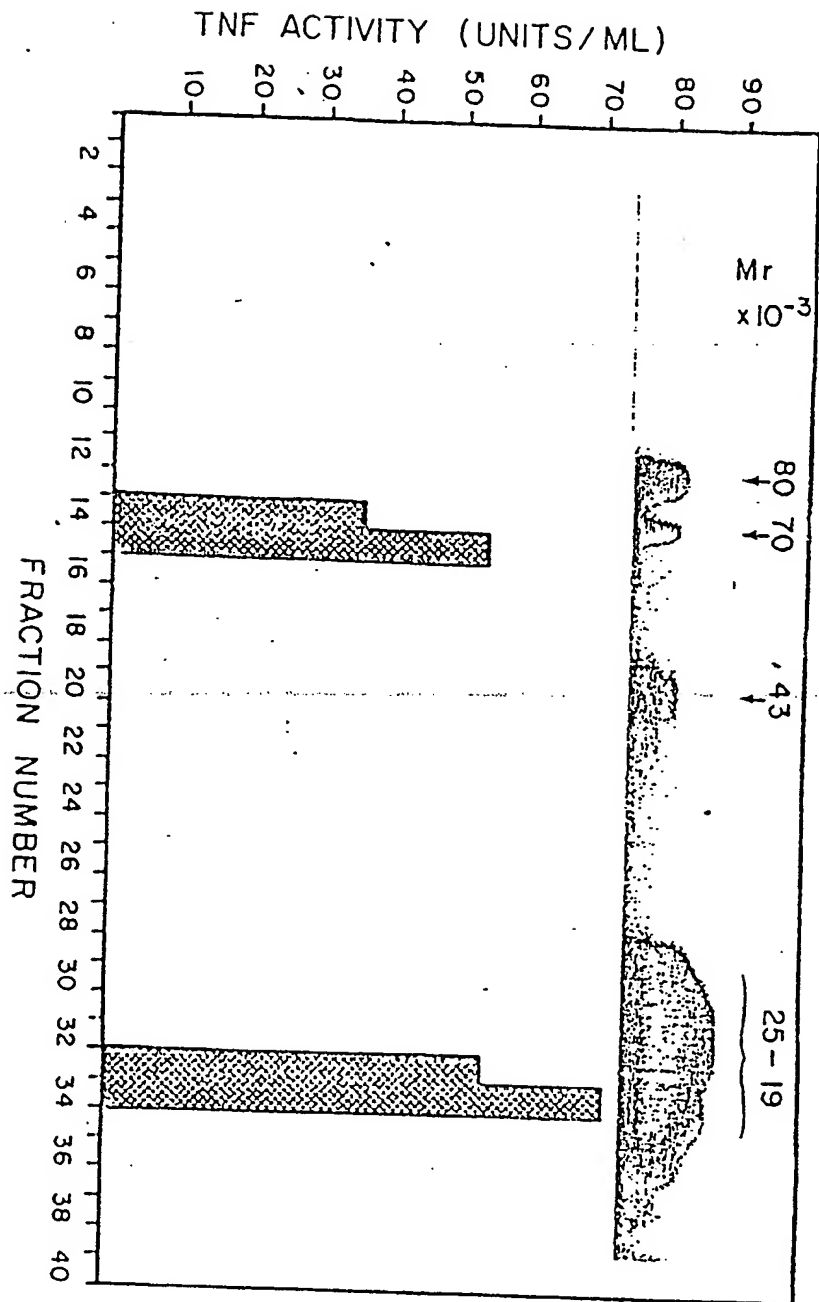
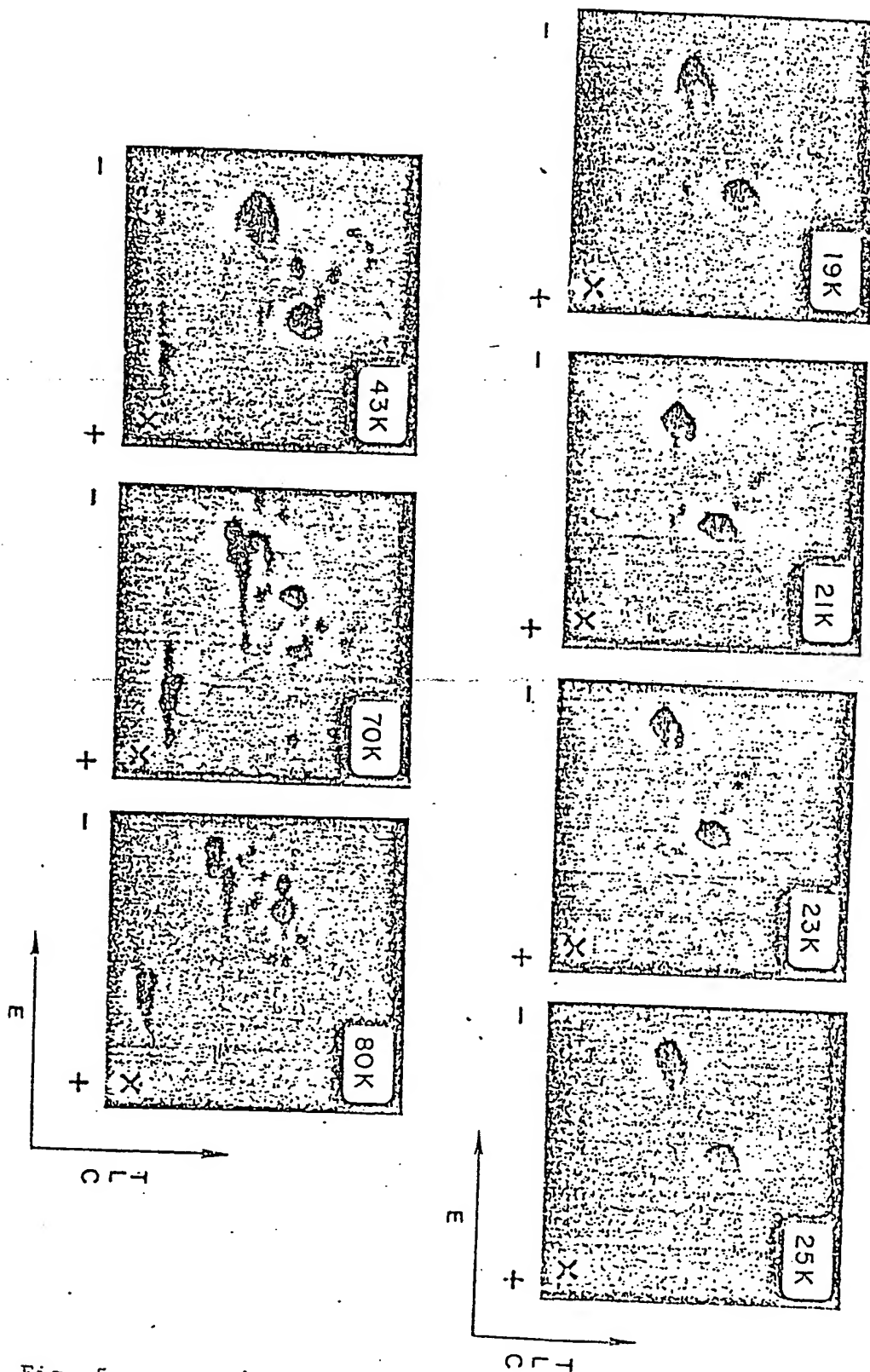


Fig. 4





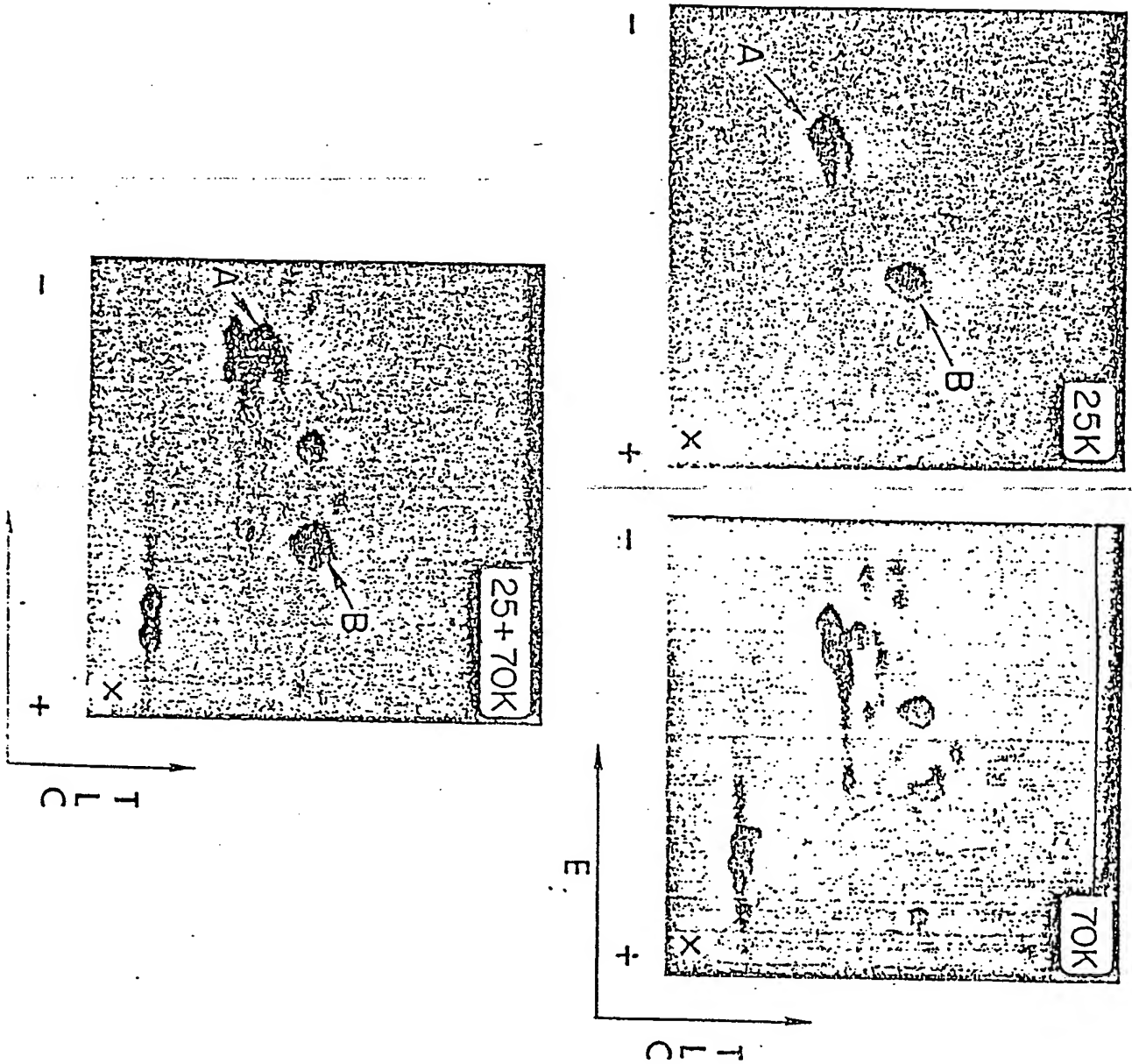


Fig. 5b

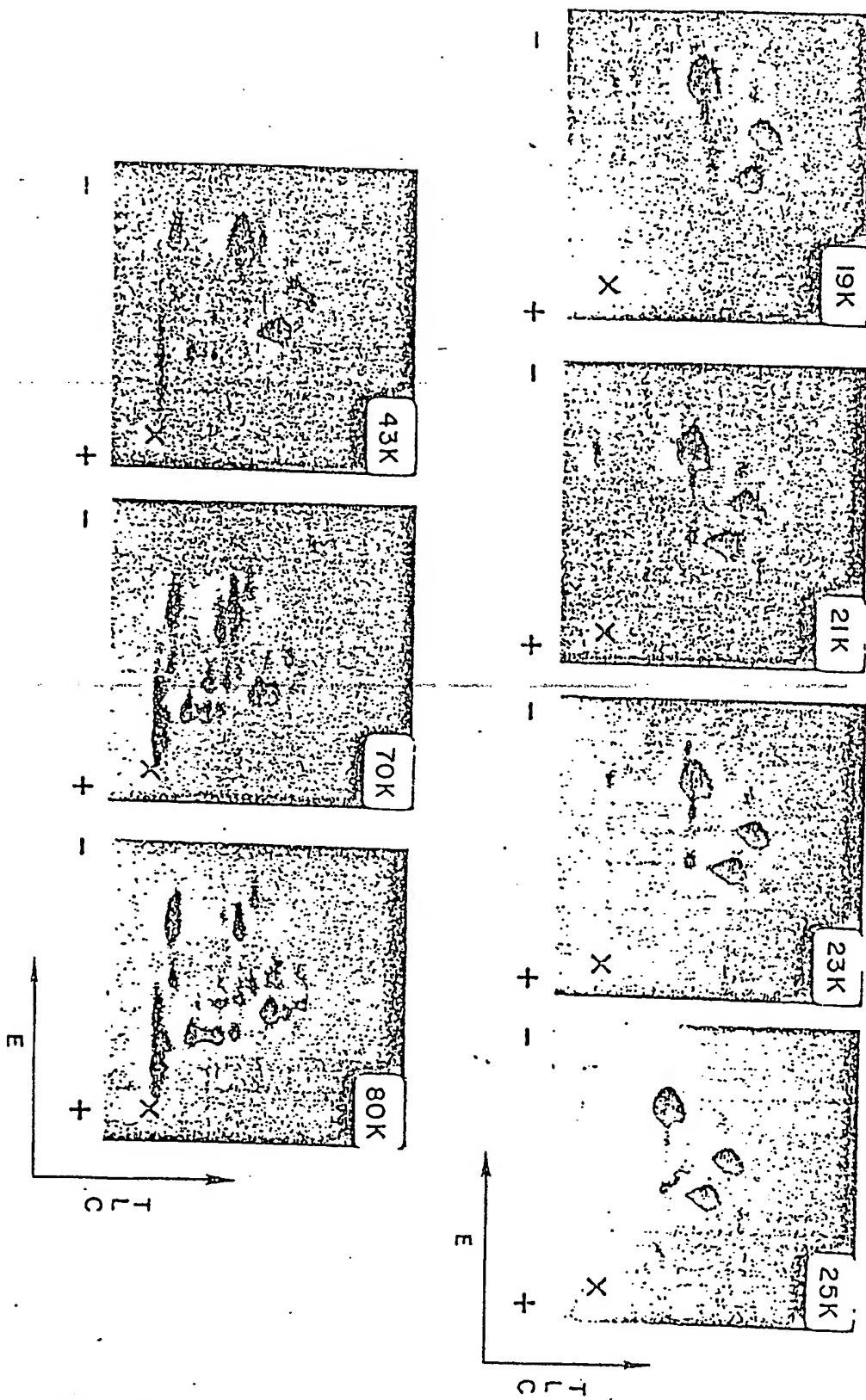


Fig. 6a

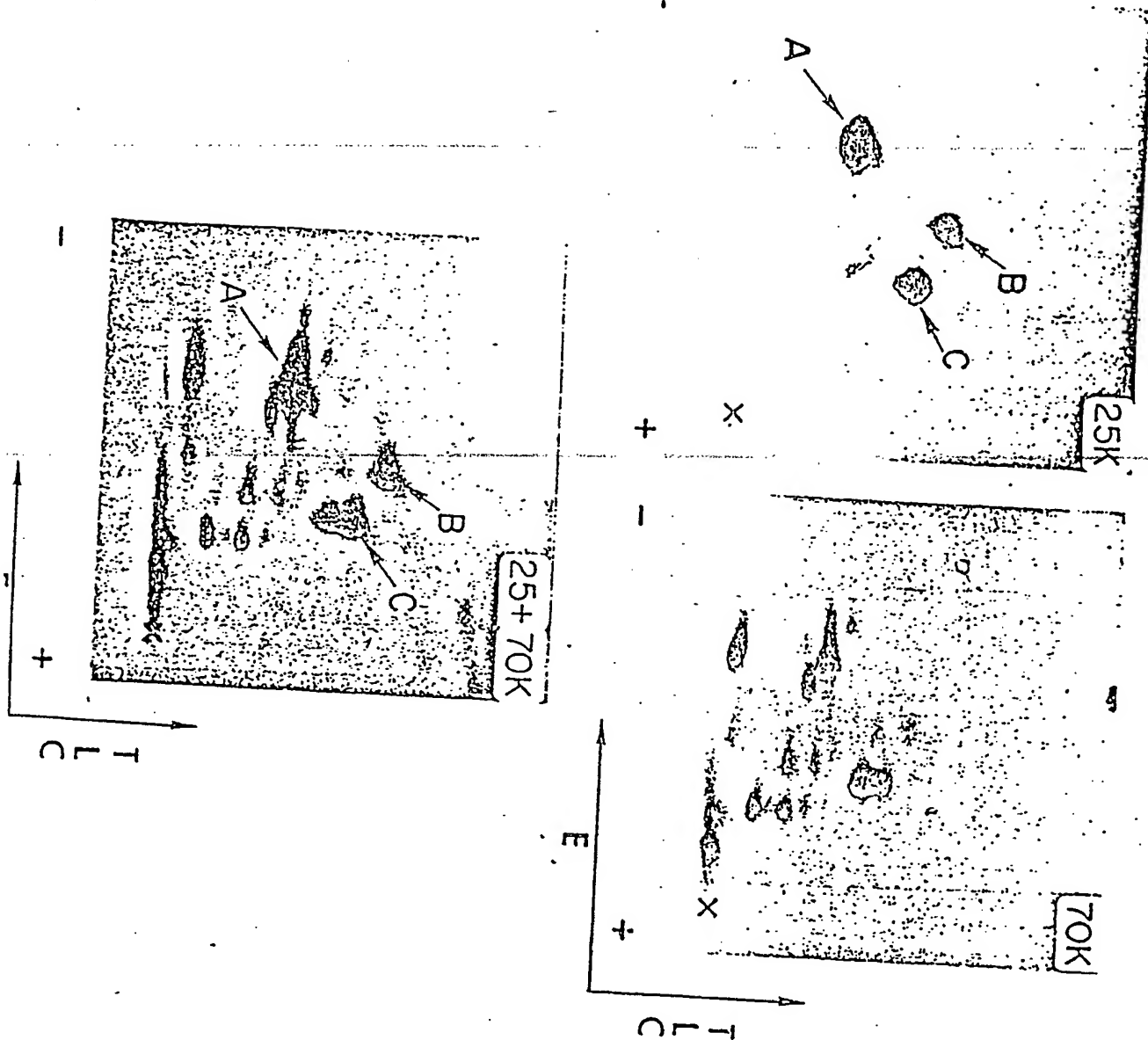


Fig. 6b

$\text{Mr} \times 10^{-3}$

43 -

19-25



Fig. 7

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EP 0 218 868 A3

㉗ Preparation of pure human tumor necrosis factor and hybridomas producing monoclonal antibodies to human tumor necrosis factor.

㉘ Human TNF(LuKII) having a specific activity of at least 1.5×10^5 units per milligram of total protein is produced by contacting a TNF-containing protein composition, which has been harvested from human cell lines of hematopoietic origin or recombinant origin, in separate adsorption stages with glass

beads, lentil lectin bound to Sepharose, and procion red agarose, thereby selectively to adsorb TNF in each stage, while leaving some impurities unadsorbed, each contact stage being followed by contact of the adsorbent with an eluant thereby to obtain a solution of more highly purified TNF after each

stage. The purified human TNF(LuKII) is used to produce monoclonal antibodies against TNF(LuKII) and such antibodies can be used to assay samples for the presence of TNF(LuKII).



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Application number

EP 86 11 1926

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 105, no. 11, September 15, 1986 page 493, abstract 95800j Columbus, Ohio, US B.Y. RUBIN et al.: "Human tumor necrosis factor (LuKII): recent developments" & ADV. IMMUNOPHARMACOL.3, Proc. Int. Conf., 3rd, 1985 (publ. 1986), 139-48 * Abstract *	1-14	C 12 P 21/02 C 12 N 5/00 C 12 P 21/00 G.01 N 33/577 A 61 K 37/24
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 260, no. 4, February 25, 1985, pages 2345-2354 B.B. AGGARWAL et al. "Human tumor necrosis factor; production, purification, and characterization" * Whole document *	1-6	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 105; no. 23, December 8, 1986, ./.		A 61 K C 12 P
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.</p> <p>Claims searched completely: 1-19 Claims searched incompletely: 20 Claims not searched: 20 Reason for the limitation of the search:</p> <p>Method for treatment of the human or animal body by surgery or therapy (See art. 52(4) of the European Patent Convention)</p>			
Place of search The Hague		Date of completion of the search 22-12-1988	Examiner CHARLES
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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
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X,P	PROC. NATL. ACAD. SCI., USA, vol. 82, October 1985, pages 6637-6641 B.Y. RUBIN et al.: "Purification and characterization of a human tumor necrosis factor from the LuKII cell line" * Whole document *	1-19	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
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Y,P	EP-A-0 183 198 (ASAHI KASEI KOGYO KABUSHIKI KAISHA) * Page 5, line 16 - page 6, line 8; page 7, line 13 - page 8, line 4; page 21, lines 6-21; claims 1,4 *	13,14	
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Y,P	DE-A- 3 421 731 (BOEHRINGER INGELHEIM INTERNATIONAL GmbH) * Page 10, paragraph 1; page 12, paragraph 2; page 20, point 2; page 23, point 4; claims 1-3 *	13,14	

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